Regulation 3.2

CSL Limited

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Immunogenic Compositions and Methods Relating Thereto"

The invention is described in the following statement:

IMMUNOGENIC COMPLEXES AND METHODS RELATING THERETO

FIELD OF THE INVENTION

The present invention relates generally to an immunogenic complex comprising a charged carrier molecule and a charged antigen and, more particularly, a negatively charged carrier molecule and a positively charged antigen. The complexes of the present invention are useful, *inter alia*, as therapeutic and/or prophylactic agents for facilitating the induction of a cytotoxic T-lymphocyte response to an antigen.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

There is an increasing belief that co-delivery of antigen and adjuvant to the same antigen-presenting-cell (APC) is preferable and sometimes essential for induction of appropriate immune responses. For example, the ability of saponin-based adjuvants to induce CD_8^+ CTL responses is attributed to their ability to cause endosomal escape of antigen, a mechanism which requires co-delivery. Particle formation which comprises a stable complex of adjuvant and antigen is the simplest way to achieve co-delivery. The usefulness of ISCOM™ technology derives partly from the immunomodulatory activity of saponins and partly from their ability to form complexes with hydrophobic or amphipathic immunogens. However, many molecules lack hydrophobic regions and in fact such molecules are preferred as recombinant proteins because of their easier expression and purification.

Accordingly, there is a need to develop immunogenic complexes which facilitate the co-delivery of antigens and carrier molecules which otherwise do not usually form sufficiently stable complexes. For example, complexes comprising antigens which lack hydrophobic regions together with adjuvant.

In work leading up to the present invention, the inventors have developed an immunogenic complex based on the electrostatic association of an antigen and a carrier molecule, such as an adjuvant. This electrostatic association permits co-delivery of the antigen and the carrier molecule to the immune system. Accordingly, by establishing an electrostatic association, antigens of interest (irrespective of their hydrophobicity) can be co-delivered with a carrier molecule, for the purpose, for example, of inducing a cytotoxic T-lymphocyte response to the antigen.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention relates to an immunogenic complex comprising a charged carrier molecule and a charged antigen which carrier molecule and antigen are electrostatically associated.

Another aspect of the present invention more particularly provides an immunogenic complex comprising a negatively charged carrier molecule and a positively charged antigen which carrier molecule and antigen are electrostatically associated.

Still another aspect of the present invention provides an immunogenic complex comprising a negatively charged carrier molecule and a positively charged protein which carrier molecule and protein are electrostatically associated.

Yet another aspect of the present invention provides an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein which adjuvant and protein are electrostatically associated.

Still yet another aspect of the present invention relates to a vaccine composition comprising as the active component an immunogenic complex comprising a charged carrier molecule and a charged antigen which carrier molecule and antigen are electrostatically associated together with one or more pharmaceutically acceptable carriers and/or diluent.

A further aspect of the present invention relates to a method of eliciting, inducing or otherwise facilitating, in a mammal, a cytotoxic T-lymphocyte response to an antigen said method comprising administering to said mammal an effective amount of a vaccine composition as hereinbefore described.

Yet another further aspect of the present invention relates to a method of treating a disease condition in a mammal said method comprising administering to said mammal an effective amount of a vaccine composition as hereinbefore described wherein administering said composition elicits, induces or otherwise facilitates an immune response which inhibits, halts, delays or prevents the onset or progression of the disease condition.

Yet another aspect of the present invention relates to the use an immunogenic complex as hereinbefore defined in the manufacture of a medicament for inhibiting, halting or delaying the onset or progression of a disease condition.

Still yet another aspect of the present invention relates to an agent for use in inhibiting, halting or delaying the onset or progression of a disease condition. Said agent comprising an immunogenic complex as hereinbefore defined.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a graphical representation of the Sucrose Gradient Analysis of HCV Core Associated ISCOMATRIX™.

Figure 1B is a graphical representation of the Sucrose Gradient Analysis of HCV Core Protein.

Figure 1C is a graphical representation of the Sucrose Gradient Analysis of ISCOMATRIX™.

Figure 2 is a graphical representation of Sucrose Gradient Analysis of E6E7 Associated ISCOMATRIX™.

Figure 3A is a graphical representation of Sucrose Gradient Analysis of NY-ESO-1 Associated ISCOMATRIX™.

Figure 3B is a graphical representation of Sucrose Gradient Analysis of NY-ESO-1 Protein.

Figure 3C is a graphical representation of Sucrose Gradient Analysis of ISCOMATRIX™.

Figure 4 is a graphical representation of Antibody Responses to NY-ESO-1 Formulations.

Figure 5A is a graphical representation of CTL Analysis of NY-ESO-1 Immunised Mice with SLLMWITQCFL Peptide.

Figure 5B is a graphical representation of CTL Analysis of NY-ESO-1 Associated ISCOMATRIX™ Immunised Mice with SLLMWITQCFL Peptide.

Figure 5C is a graphical representation of CTL Analysis of NY-ESO-1 Immunised Mice with SLLMWITQC Peptide.

Figure 5D is a graphical representation of CTL Analysis of NY-ESO-1 Associated ISCOMATRIX™ Immunised Mice with SLLMWITQC Peptide.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated, in part, on the development of an immunogenic complex formulation which utilises electrostatic interactions to associate an antigen and a carrier molecule thereby facilitating, *inter alia*, the co-delivery of these molecules to the immune system. The immunogenic complexes of the present invention are particularly suitable for use in facilitating the stimulation of cytotoxic T-lymphocyte responses to immunogens which do not comprise hydrophobic regions.

Accordingly, one aspect of the present invention relates to an immunogenic complex comprising a charged carrier molecule and a charged antigen which carrier molecule and antigen are electrostatically associated.

Reference to a "charged" carrier molecule or antigen should be understood as a reference to a carrier molecule or antigen which exhibits an overall positive electrical charge or an overall negative electrical charge. By "overall" is meant the summation of the individual positive and negative charges which a given molecule comprises. Where the summation of the individual positive and negative charges results in overall electrical neutrality, the molecule is not regarded as "charged" within the context of the present invention. Preferably, the antigen comprises an overall positive charge and the carrier molecule comprises an overall negative charge.

Accordingly, the present invention more particularly provides an immunogenic complex comprising a negatively charged carrier molecule and a positively charged antigen which carrier molecule and antigen are electrostatically associated.

Reference to "electrostatically associated" is a reference to the carrier molecule and the antigen being linked, bound or otherwise associated by means which include electrostatic interaction. Accordingly, it should be understood that in some instances the electrostatic interaction will be the only attractive force which results in complexing of the antigen and the

carrier molecule. However, in other instances the formation of the electrostatic interaction may also lead to, or be associated with, the formation of other interactive forces.

Reference to "antigen" should be understood as a reference to any molecule against which it is sought to induce an immune response, and in particular, a cytotoxic T-lymphocyte response. The antigen may be either a proteinaceous or a non-proteinaceous molecule, which molecule may or may not be immunogenic if it were administered in isolation. The antigen of the present invention may be naturally derived or it may be recombinantly or synthetically produced. Following its isolation or synthesis the antigen may require further modification (for example, structural or sequence modification to improve its antigenicity) prior to use in the present invention. Antigens suitable for use in the present invention include, but are not limited to, core proteins isolated from viruses, non-core viral proteins, antigens of malignant and non-malignant cells, bacterial antigens and parasite antigens.

Preferably, the antigen is a protein. The term "protein" should be understood to encompass reference to proteins, polypeptides and peptides. The protein may be glycosylated or unglycosylated, phosphorylated or dephosphorylated to various degrees and/or may contain a range of other molecules fused, linked, bound or otherwise associated to the protein such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. Reference hereinafter to a "protein" includes a protein comprising a sequence of amino acids as well as a protein associated with other molecules such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins.

According to this preferred embodiment, there is provided an immunogenic complex comprising a negatively charged carrier molecule and a positively charged protein which carrier molecule and protein are electrostatically associated.

In this regard, the antigen which is included in the immunogenic complex of the present invention may be, in its initial or natural form, positively charged, negatively charged or of neutral charge. Where an antigen is positively charged, it may nevertheless be weakly

positively charged and may therefore require modification to increase its degree of positive charge such that complex formation with the negatively charged carrier molecule is better facilitated. For example, wherein an antigen is weakly positively charged, increasing the degree of its positive charge may be achieved by any one of a number of methods known to those skilled in the art including, but not limited to, chemically adding further positive charge to the antigen or recombinantly adding positive charge such as by adding polylysine to the antigen. This is of particular use where the antigen is a protein. Other methods which may be utilised to increase the degree of an antigen's positive charge include, but are not limited to, pH modification, chemical modifications or neutralisation of an antigen's negative charges with positively charged molecules such as arginine. Similarly, where an antigen is neutral or negatively charged, its overall charge can be converted to an overall positive charge by utilising such methodology. Conversion of a negatively charged antigen to express an overall positive charge may be of particular importance where the antigen is a protein, since most proteins are naturally negatively charged.

Once the charge of the antigen of interest is sufficiently positive, it becomes necessary to ensure that precipitation of the positively charged antigen does not occur prior to complex formation with the carrier molecule. In this regard, any suitable method for preventing antigen precipitation may be utilised. For example, antigen solubility may be maintained by disrupting the forces that cause antigen aggregation. Disruption of these forces can be achieved, for example, by incorporating into the antigen solution chaotrophic agents such as urea and guanidine, solvents such as DMSO (dimethyl sulfoxide) and acetonitrile, intermediates such as zwitterions, detergents such as Triton X-100 and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate), reducing agents such as DTT (dithiothreitol) and cysteine and chelating agents such as EDTA (ethylene diaminetetraacetic acid). Solubility can also be maintained by altering the pH of the antigen solution or by chemical modification of the antigen to introduce polar or ionic molecules such as by alkylation or acetylation.

Reference to "carrier molecule" should be understood as a reference to any molecule which, when an antigen is associated with it, facilitates the induction of an immune response, and in particular a cytotoxic T-lymphocyte response, to the antigen. In a particularly preferred embodiment the carrier molecule is an adjuvant. By "adjuvant" is meant any molecule or any aggregate or complex of molecules which functions to stimulate, enhance or otherwise up-regulate any one or more aspects of the immune response. For example, the adjuvant may induce inflammation thereby attracting immune response cells to the site of antigen localisation. Alternatively, the adjuvant may slowly release the antigen thereby providing on-going stimulation of the immune system. Examples of adjuvants suitable for use in the present invention include, but are not limited to, saponin, saponin complexes, any one or more components of the immunostimulating complex of saponin, cholesterol and lipid known as ISCOMATRIX™ (for example the saponin component and/or the phospholipid component), liposomes or oil-in-water emulsions. [The composition and preparation of ISCOMATRIX™ is described in detail in International Patent Application Number PCT/SE86/00480, Australian Patent Numbers 558258 and 632067 and European Patent Publication No. 0 180 564, the disclosures of which are incorporated herein by reference]. Further examples of adjuvants include, but are not limited to, those detailed in the publication of Cox and Coulter, 1992.

Accordingly, the present invention still more preferably provides an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein which adjuvant and protein are electrostatically associated.

Preferably, said adjuvant is saponin or a saponin complex. More preferably, said saponin complex is ISCOMATRIX™.

The carrier molecule of the present invention may also be, in its initial or natural form, negatively charged, positively charged or neutral. Increasing the degree of negative charge (for example, where the carrier molecule is only weakly negatively charged) or converting a neutral or positively charged carrier molecule to a negatively charged carrier molecule may

also be achieved by any suitable method known to those skilled in the art. For example, where the carrier is an oil-in-water emulsion, incorporation of any anionic surfactant with a non-polar tail will impart an overall negative charge to the emulsion due to insertion of the tail of the surface of the oil droplet which thereby leaves the negatively charged head group exposed. The negative charge of a saponin complex adjuvant may be increased, for example, by the addition of negatively charged lipid during complex formation.

The present invention is predicated, in part, on the formation of immunogenic complexes via the electrostatic association, preferably, of a negatively charged carrier molecule with a positively charged antigen. The administration of such a complex to a subject facilitates the induction of a significantly better immune response than would be achieved were the adjuvant and antigen administered simultaneously but in a non-associated form. In particular, the administration of an antigen associated with an adjuvant, according to the present invention, facilitates the induction of a cytotoxic T-lymphocyte response to the antigen. However, humoral and other cellular responses can also be enhanced.

Without limiting the present invention to any one theory or mode of action, it is thought that the complexing of the adjuvant with the antigen facilitates co-delivery of the adjuvant and the antigen to the same antigen presenting cell thereby facilitating the induction of immune responses which either would not occur or would not occur as effectively were these molecules not co-delivered. For example, the induction of some CD8+ cytotoxic T-lymphyocyte responses are thought to occur where the adjuvant induces endosomal escape of the antigen in the antigen presenting cell. This necessarily requires co-delivery of the antigen and the adjuvant to the antigen presenting cell.

A further aspect of the present invention therefore relates to the use of the invention to induce an immune response in a mammal including, but not limited to, a humoral and/or cell mediated immune response.

Accordingly, another aspect of the present invention relates to a vaccine composition comprising as the active component an immunogenic complex comprising a charged carrier molecule and a charged antigen which carrier molecule and antigen are electrostatically associated together with one or more pharmaceutically acceptable carriers and/or diluent.

Preferably, said carrier molecule is an adjuvant, and even more preferably saponin or a saponin complex. Preferably said saponin complex is ISCOMATRIX™.

Still more preferably, said antigen is a protein.

Preferably said carrier molecule is negatively charged and said antigen is positively charged.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to

materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Without limiting the operation of the present invention in any way, the co-delivery of the immunogenic complex of the present invention is particularly useful for inducing a cytotoxic T-lymphocyte response to an antigen.

Accordingly, still another aspect of the present invention relates to a method of eliciting, inducing or otherwise facilitating, in a mammal, a cytotoxic T-lymphocyte response to an antigen said method comprising administering to said mammal an effective amount of a vaccine composition as hereinbefore described.

A further aspect of the present invention relates to the use of the immunogenic complex of the invention in relation to the therapeutic and/or prophylactic treatment of disease conditions. Examples of disease conditions which can be treated in accordance with the method of the present invention include, but are not limited to, HIV, Hepatitis B, Hepatitis C, melanoma, prostate cancer, breast cancer, tuberculosis and parasitic conditions.

Accordingly, yet another aspect of the present invention relates to a method of treating a disease condition in a mammal said method comprising administering to said mammal an effective amount of a vaccine composition as hereinbefore described wherein administering said composition elicits, induces or otherwise facilitates an immune response which inhibits, halts, delays or prevents the onset or progression of the disease condition.

An "effective amount" means an amount necessary at least partly to attain the desired immune response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The term "mammal" includes humans, primates, livestock animals (eg. horses, cattle, sheep, pigs, donkeys), laboratory test animals (eg. mice, rats, rabbits, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. kangaroos, deer, foxes). Preferably, the mammal is a human or laboratory test animal. Even more preferably, the mammal is a human.

The mammal undergoing treatment may be human or an animal in need of therapeutic or prophylactic treatment of a disease condition or a potential disease condition.

In yet another aspect the present invention relates to the use an immunogenic complex as hereinbefore defined in the manufacture of a medicament for inhibiting, halting or delaying the onset or progression of a disease condition.

Yet another aspect of the present invention relates to an agent for use in inhibiting, halting or delaying the onset or progression of a disease condition. Said agent comprising an immunogenic complex as hereinbefore defined.

Further features of the present invention are more fully described in the following Figures and/or Examples.

Reference to "ISCOPREP™ 703" (referred to hereinafter as "703") should be understood as a reference to a saponin preparation comprising from 50-90% by weight of Fraction A of Quil A and 50% to 10% by weight of Fraction C of Quil A. Fractions A and C are prepared from the lipophilic fraction of Quil A. Fractions "A" and "C", their method of preparation and the method of preparing 703 are detailed in International Patent Publication No. WO96/11711, which is incorporated herein by reference.

EXAMPLE 1

PREPARATION OF ISCOMATRIX™ (Immunostimulating complexes without immunogen)

ISCOMATRIX™ (Immunostimulating complex without antigen) was prepared essentially by the method of Morein *et al.* (1989). Briefly, to 1.76 ml PBS pH 7.2 was added 0.16 ml of a solution containing 10 mg/ml cholesterol and 10 mg/ml dipalmitylphosphatidylcholine (DPPC) in 20% MEGA-10 detergent (w/v) then 0.08 ml of a solution containing 100 mg/ml 703 in PBS. The solution was held at 25°C for 1 hour with gentle mixing. During subsequent dialysis against PBS/azide, immunostimulating complexes containing cholesterol, DPPC and 703 were formed. This ISCOMATRIX™ was of typical appearance by electron microscopy.

EXAMPLE 2

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A NATURALLY POSITIVELY CHARGED PROTEIN : HCV CORE PROTEIN

The HCV Core protein has a pI of 11.4 making it a very positively charged protein at pH7.2. Solubility of the HCV core was maintained using 30mM Citrate, 0.23M NaCl, 1mM EDTA, 0.01% Tween 80 at pH5. The HCV Core associated ISCOMATRIX™ formulation was prepared by mixing equal mass of HCV Core and 703 as ISCOMATRIX™ for 60 minutes at 20-25°C.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for protein, association between Core and ISCOMATRIX™ and ISCOMATRIX™ (Figure 1). Protein was detected using the Pierce Coomassie Plus assay according to the manufacturers instructions. Association was determined by EIA using a monoclonal antibody to Core to capture and a HRP conjugate monoclonal antibody to 703 to detect (referred to herein as the "703 assay"). ISCOMATRIX™ was determined by detection of 703 which was assayed for by coating fractions to a microtitre plate then detecting with the HRP conjugated monoclonal antibody to 703.

The HCV Core protein, when not mixed with ISCOMATRIX™, was found in fractions 9-12 by the Pierce assay, with negative readings for the association and 703 assays (Figure 1B). ISCOMATRIX™ alone was found in fractions 5-8 by the 703 assay with negative readings in the Pierce and association assays (Figure 1C). The mixture of HCV Core protein and ISCOMATRIX™ showed that both protein and ISCOMATRIX™ were found in the same fractions by the Pierce, association and 703 assays (Figure 1A) indicating association had occurred.

EXAMPLE 3

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A PROTEIN UTILISING pH TO GIVE A POSITIVE CHARGE : HPV E6E7.

The HPV E6E7 fusion protein has a pI of 5.9 making it a negatively charged protein at pH7.2. It contains a hexa histidine sequence at the N terminus which will be positively charged at pH6. Solubility of the E6E7 was maintained using 8M urea, 50mM Bis Tris, 0.15M NaCl pH6. The E6E7 associated ISCOMATRIX™ formulation was prepared by mixing equal mass of E6E7 antigen and ISCOMATRIX™ for 60 minutes at 20-25 C, dialysing against 50mM Bis Tris, 0.15M NaCl pH6 to remove the urea then centrifugationat 10,000 g for 5 mins to remove any precipitate.

After formulation, preparations were purified on a sucrose gradient (50 to 10% sucrose w/v) and fractions analysed for protein, association between E6E7 and ISCOMATRIX™ and ISCOMATRIX™ (Figure 2). Protein was detected using a sandwich EIA for E7 with non-competiting monoclonal antibodies to capture and detect. Association was determined by EIA using a monoclonal antibody to E7 to capture and a HRP conjugated monoclonal antibody to 703 to detect. ISCOMATRIX™ was determined by detection of dipalmityl phosphatidyl choline (DPPC) using diphenylhexatriene (DPH). Briefly, DPH is dissolved at 1mg/ml in acetone then diluted 1 in 50 in PBS 0.1% azide pH7.2 then 50μl mixed with 50μl of each fraction in a microtitre plate. Following incubation for 150 mins at 20-25°C the plate is read in a microplate fluorometer using excitation 355nm and emission 460nm.

The E6E7 protein was largely confined to fractions 10-15, as measured by the E7 EIA, as was association of E6E7 and 703, as measured by EIA, and approximately half the DPPC, as measured by DPH, indicating association between E6E7 and ISCOMATRIX™ . Typically E6E7 protein is found in fractions 2-5 and ISCOMATRIX™ in fractions 10-15 when run separately.

EXAMPLE 4

IMMUNIZATION OF MICE WITH E6E7 ASSOCIATED ISCOMATRIX™.

Six C57Bl6 mice were immunized, on days 0 and 21, subcutaneously in the scruff of the neck with 0.1 ml of E6E7 associated ISCOMATRIX™ containing 6μg 703 and 6μg E6E7.

Antibody Responses:

Mice were bled on day 26 and sera analysed for antibodies to E7 by indirect EIA. Purified GSTE7 was adsorbed to a microtitre plate in 0.1M Carbonate pH9.6 then the plate blocked with a 0.1% casein solution and dried. Dilutions of sera were incubated for 1 hour at 20-25°C then the plates washed. HRP conjugated goat α mouse IgG was added and plates incubated for 1 hour at 20-25°C then washed. TMB substrate was added and incubated for

10 mins at 20-25°C followed by addition of 0.5M H_2SO_4 to stop the reaction. Plates were read at OD450nm and end point titres calculated.

The E6E7 associated ISCOMATRIX™ group had a GMT of 949. Typically E6E7 with $Al(OH)_3$ gives a GMT of approximately 100.

Cytokine Responses:

On day 27 splenocytes from each of 3 mice were harvested and pooled and $2.5X10^6$ cells restimulated in 48-well plates with GSTE7 at 1 and 5 μg and ConA and RPMI as controls. Cells were cultured in RPMI media supplemented with 10% foetal calf serum, 2mM glutamine, $5X10^{-5}$ Mβ-mercaptoethanol, 100μg/ml streptomycin and 100IU/ml pencillin and incubated at 37°C for 2 days in 5%CO_2. The supernatant was harvested and γIFN and IL5 detected by EIA using reagents from Endogen according to manufacturers instructions.

The E6E7 associated ISCOMATRIX™ induced up to 7.4 ng/ml γIFN and 140 pg/ml IL5 (Table 1). Typically E6E7 with $Al(OH)_3$ induces no detectable γIFN (<30 pg/ml) or IL5 (<4 pg/ml).

EXAMPLE 5

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A NATURALLY POSITIVELY CHARGED PROTEIN : NY-ESO-1 PROTEIN

The NY-ESO-1 protein has a pI of 9.1 making it a very positively charged protein at pH7.2. Solubility of the NY-ESO-1 was maintained using 8M urea. The NY-ESO-1 associated ISCOMATRIX™ formulation was prepared by mixing equal mass of NY-ESO-1 and ISCOMATRIX™ and mixing for 60 minutes at 20-25°C.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for protein, association between NY-ESO-1 and ISCOMATRIX™ and

ISCOMATRIX™ (Figure 3). Protein was detected by EIA using a HRP conjugated monoclonal antibody to NY-ESO-1 to detect fractions adsorbed to a microtitre plate. Association was determined by EIA using a monoclonal antibody to NY-ESO-1 to capture and a HRP conjugated monoclonal antibody to 703 to detect. ISCOMATRIX™ was determined by detection of 703 which was assayed for by coating fractions to a microtitre plate then detecting with the HRP conjugated monoclonal antibody to 703. The NY-ESO-1 protein not mixed with ISCOMATRIX™ was found in fractions 1-5 by protein EIA with negative readings for the association and 703 assays (Figure 3B). ISCOMATRIX™ was found in fractions 4-8 by the 703 assay with negative readings in the protein and association assays (Figure 3C). The mixture of NY-ESO-1 and ISCOMATRIX™ showed protein and 703 in fractions 6-9 by protein, association and 703 EIA's (Figure 3A) indicating association had occurred.

EXAMPLE 6

IMMUNISATION OF MICE WITH NY-ESO-1 FORMULATIONS

Antibody Responses:

Ten BALB/c mice were immunised, on days 0 and 28, subcutaneously in the scruff of the neck with 0.1ml of NY-ESO-1 containing 5μg protein or NY-ESO-1 associated ISCOMATRIX™ containing 5μg protein and 5μg QH. The mice were bled on day 35 and the sera analysed for antibodies to NY-ESO-1 by indirect EIA. The NY-ESO-1 was adsorbed to a microtitre plate in PBS pH7.2 then the plate blocked with a 0.1% casein solution and dried. Dilutions of sera were incubated for 1 hour at 20-25°C then the plates washed. HRP conjugated goat α mouse IgG, IgG_1 or IgG_{2a} was added and plates incubated for 1 hour at 20-25°C then washed. TMB substrate was added and incubated for 10 mins at 20-25°C followed by addition of 0.5M H_2SO_4 to stop the reaction. Plates were read at OD450nm and end point titres calculated.

There was a greater than 20 fold increase in the IgG and IgG_1 responses to NY-ESO-1 when associated with ISCOMATRIX™. Very little IgG_{2a} was induced with NY-ESO-1 alone but when associated with ISCOMATRIX™ there was a thousand fold increase in IgG_{2a} titre.

Cytotoxic T Lymphocyte (CTL) Responses:

Five HLA A2 transgenic HHD mice were immunised subcutaneously at the base of the tail with 0.1ml of NY-ESO-1 containing 5μg protein or NY-ESO-1 associated ISCOMATRIX™ containing 5μg protein and 5μg QH. After 14 days splenocytes were harvested and $5X10^6$ cells restimulated in 24-well plates with EL4HHD cells sensitised with NY-ESO-1 peptide (10μg/ml for 1 hour 37°C), irradiated and washed twice. Cells were cultured in RPMI media supplemented with 10% foetal calf serum, 2mM glutamine, $5X10^{-5}$ Mβ-mercaptoethanol, 100μg/ml streptomycin and 100IU/ml pencillin and incubated at 37°C for 6 days in 5%CO_2. On day 4 1ml of medium was added containing 5U/ml recombinant human IL-2. On day 6 the cultures were used as effectors in standard 6 hour ^{51}Chromium release assays against EL4HHD cells sensitised as for restimulation.

CTL were not detected in mice immunised with NY-ESO-1 alone but when associated with ISCOMATRIX™ , CTL were detected in all mice (Figure 5).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 1 Cytokine Analysis of E6E7 Associated ISCOMATRIX™

Stimulated with	Concentration µg	Cytokine pg/ml	
		γIFN	IL5
GSTE7	5	7400	140
GSTE7	1	1050	85
ConA	0.4	2130	74
RPMI	-	<30	4

REFERENCES

Morein, B., Lövgren, K. and Hoglund, S., 1989, Immunostimulating complex (ISCOM). In "Vaccines: Recent Trends and Progress." G. Gregoriadis, A.C. Allison and G. Poster (Eds), Plenium Press, New York, p153.

Cox J.C. and Coulter A.R. *Advances in Adjuvant Technology and Application in Animal Parasite Control Utilising Biotechnology*. Chapter 4. Editor Yong, W.K. CRC Press, 1992.

Figure 1. Sucrose Gradient Analysis of HCV Core Formulations

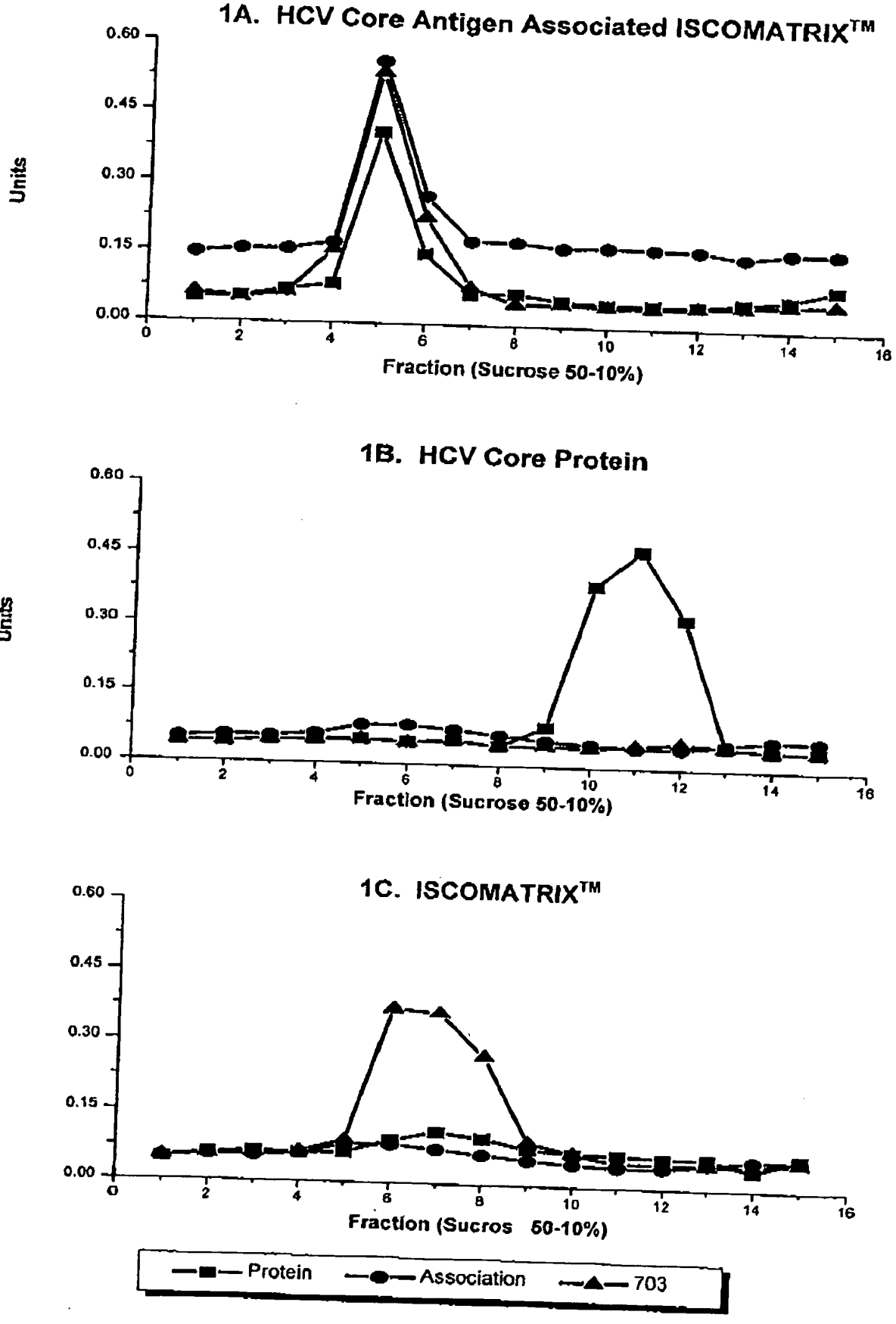


Figure 2. Sucrose Gradient Analysis of E6E7 Associated ISCOMATRIX™

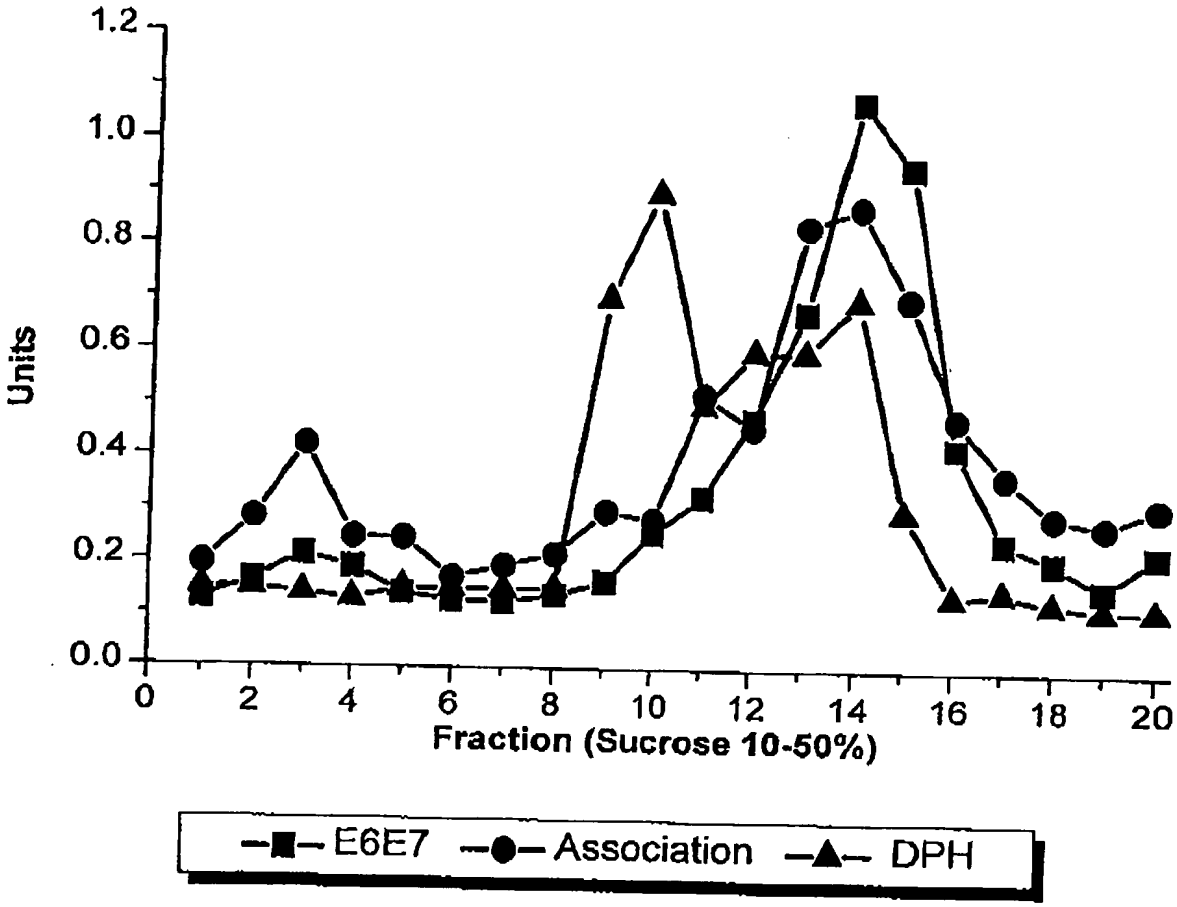


Figure 3. Sucrose Gradient Analysis of NY-ESO-1 Formulations

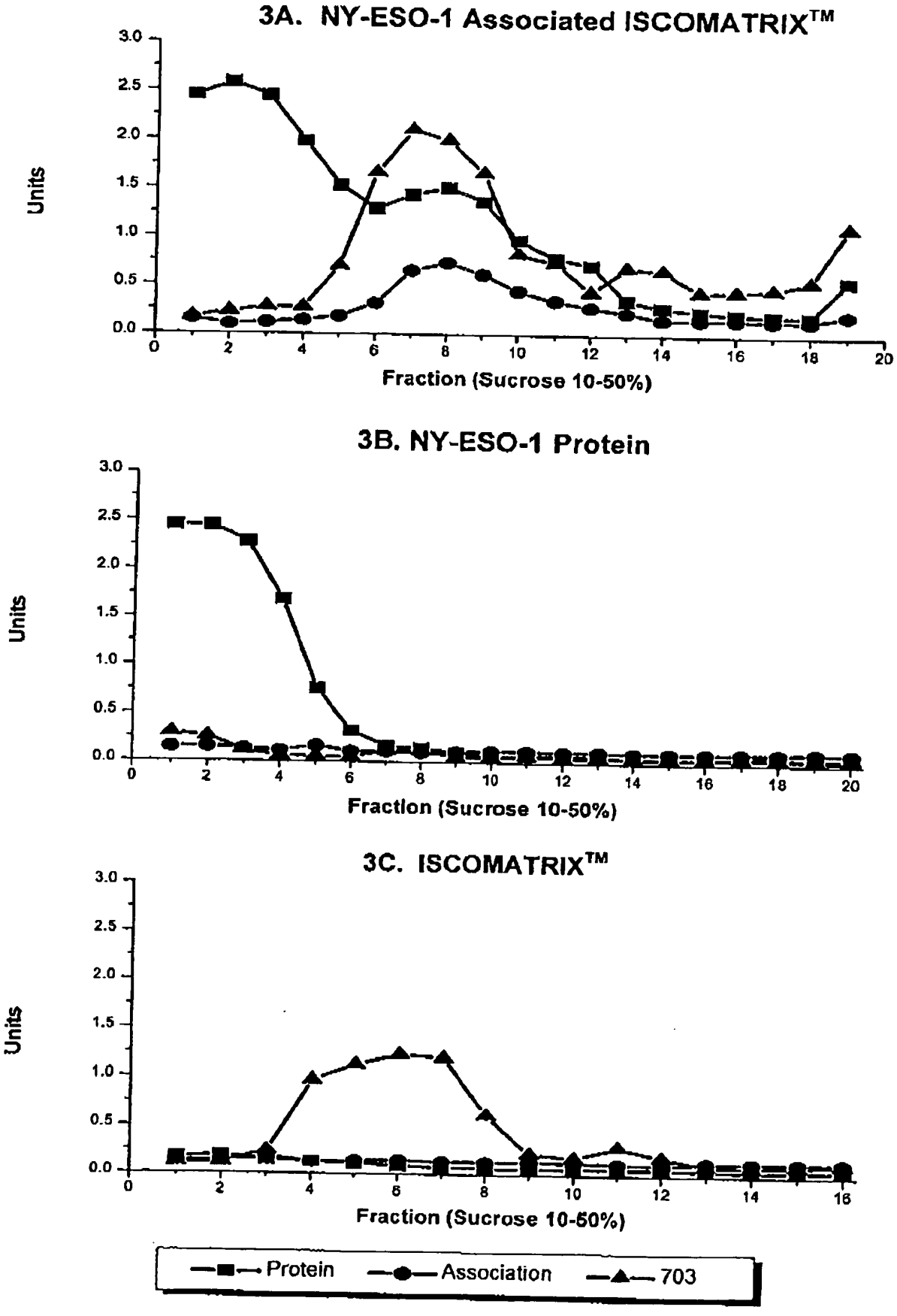


Figure 4. Antibody Response to NY-ESO-1 Formulations

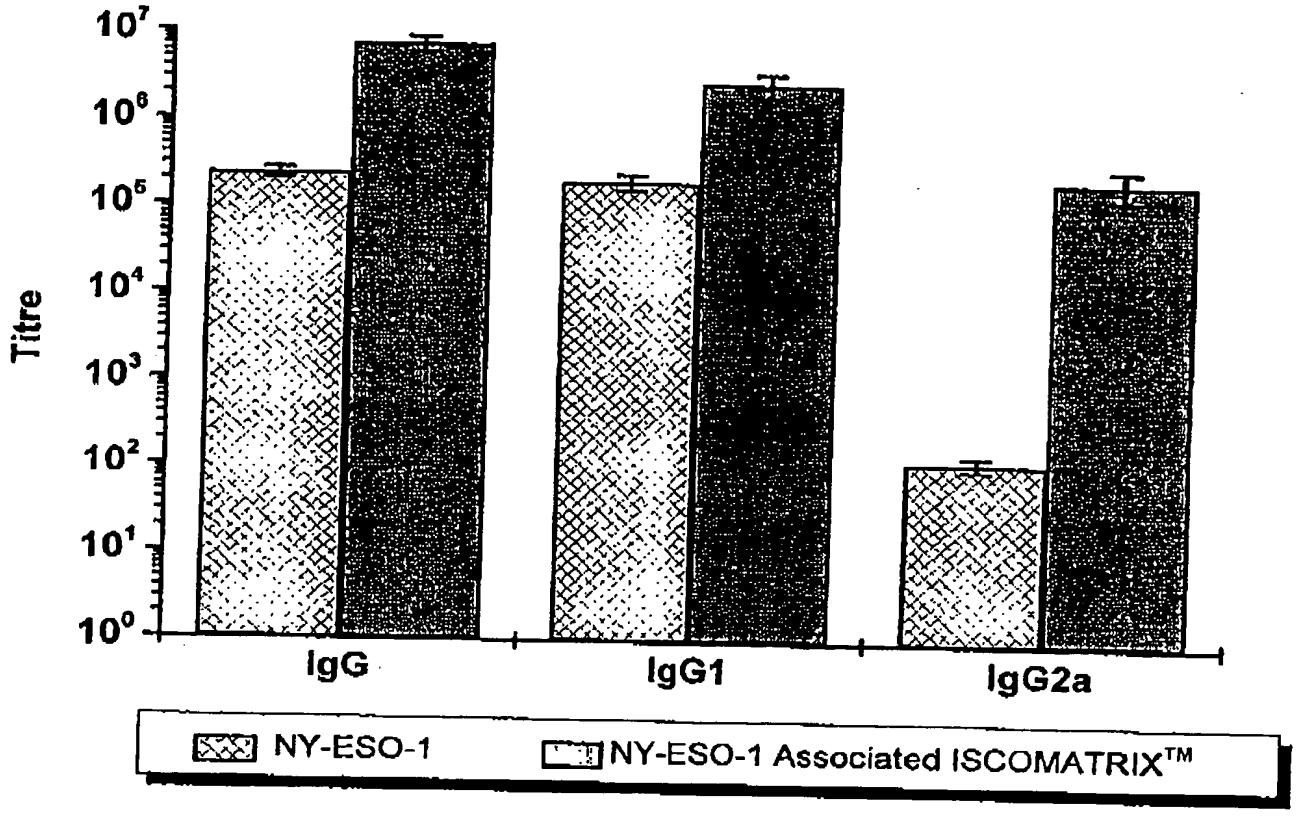
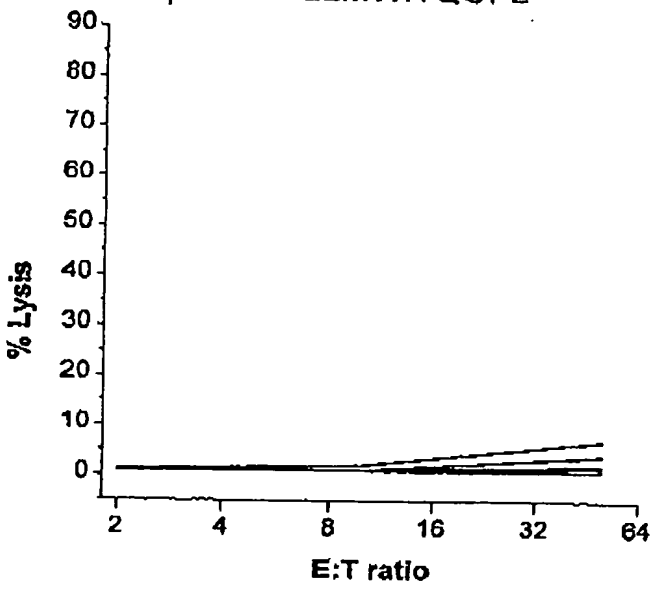


Figure 5. CTL Analysis of NY-ESO-1 Formulations

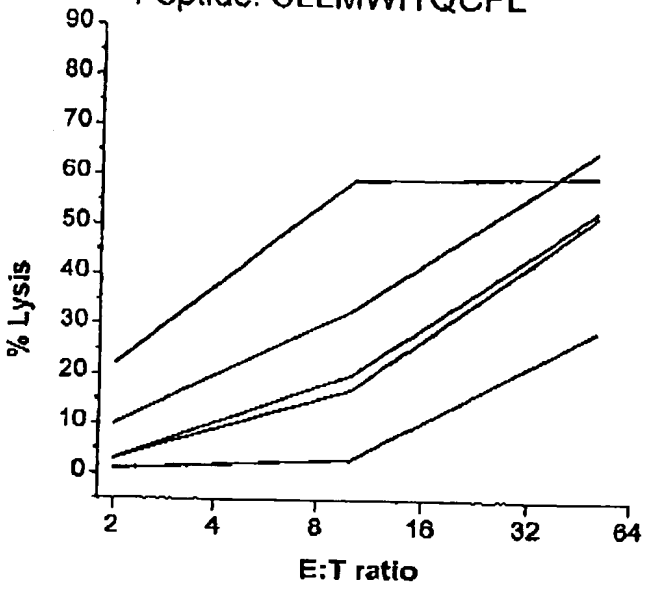
5A. NY-ESO-1

Peptide : SLLMWITQCFL



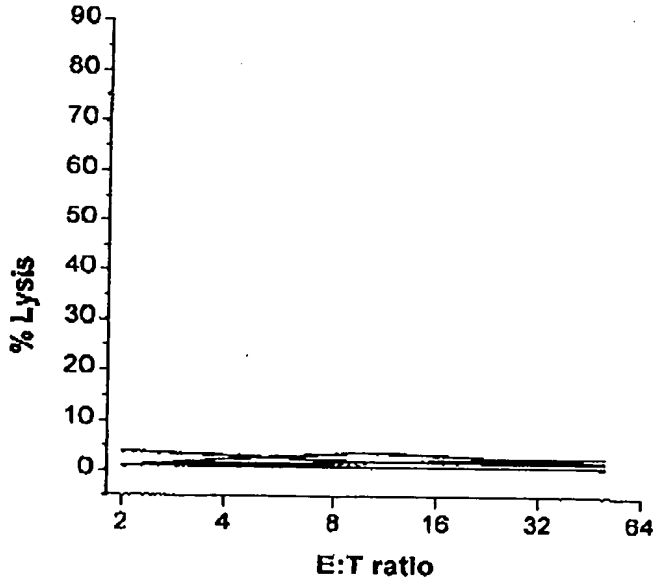
5B. NY-ESO-1 Associated ISCOMATRIX™

Peptide: SLLMWITQCFL



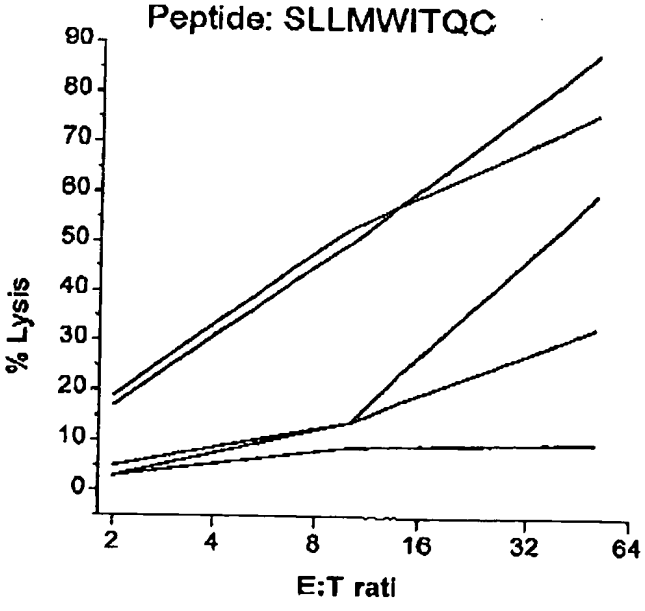
5C. NY-ESO-1

Peptide: SLLMWITQC



5D. NY-ESO-1 Associated ISCOMATRIX™

Peptide: SLLMWITQC



US 0950601105P1

Creation date: 05-25-2004
Indexing Officer: TROBINSON - TERRI ROBINSON
Team: OIPEBackFileIndexing
Dossier: 09506011

Legal Date: 08-16-2000

No.	Doccode	Number of pages
1	LET.	1

Total number of pages: 1

Remarks:

Order of re-scan issued on .....